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Influence of Membrane Lipid Composition on a Transmembrane Bacterial Chemoreceptor*

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Divya N. Amin and Gerald L. Hazelbauer[1]

From the Department of Biochemistry, University of Missouri, Columbia, Missouri 65211

Background: Nothing was known about influences of membrane lipids on transmembrane chemoreceptors.
Results: Assays of adaptational modification of chemoreceptors in Nanodiscs containing different lipids revealed functionally native structure depended on lipid composition.
Conclusion: Chemoreceptors are strongly influenced by lipid environment and tuned to their natural one.
Significance: Lipids surrounding the small portion of a chemoreceptor in the membrane have profound effects on functional structure.

Most bacterial chemoreceptors are transmembrane proteins. Although less than 10% of a transmembrane chemoreceptor is embedded in lipid, separation from the natural membrane environment by detergent solubilization eliminates most receptor activities, presumably because receptor structure is perturbed. Reincorporation into a lipid bilayer can restore these activities and thus functionally native structure. However, the extent to which specific lipid features are important for effective restoration is unknown. Thus we investigated effects of membrane lipid composition on chemoreceptor Tar from *Escherichia coli* using Nanodiscs, small (~10-nm) plugs of lipid bilayer rendered water-soluble by an annulus of "membrane scaffold protein." Disc-enclosed bilayers can be made with different lipids or lipid combinations. Nanodiscs carrying an inserted receptor dimer have high protein-to-lipid ratios approximating native membranes and in this way mimic the natural chemoreceptor environment. To identify features important for functionally native receptor structure, we made Nanodiscs using natural and synthetic lipids, assaying extents and rates of adaptational modification. The proportion of functionally native Tar was highest in bilayers closest in composition to *E. coli* cytoplasmic membrane. Some other lipid compositions resulted in a significant proportion of functionally native receptor, but simply surrounding the chemoreceptor transmembrane segment with a lipid bilayer was not sufficient. Membranes effective in supporting functionally native Tar contained as the majority lipid phosphatidylethanolamine or a related zwitterionic lipid plus a rather specific proportion of anionic lipids, as well as unsaturated fatty acids. Thus the chemoreceptor is strongly influenced by its lipid environment and is tuned to its natural one.

The majority of bacterial chemoreceptors are transmembrane proteins (1). Among transmembrane chemoreceptors, the most common organization (1) is exemplified by the well studied chemoreceptors of *Escherichia coli* and *Salmonella* (2), which span the membrane with a loosely packed four-helix bundle (3, 4) that is less than 10% of the entire receptor (see Fig. 1, *A* and *B*). When such receptors are removed from their natural lipid environment by detergent solubilization, they lose most activities (5, 6), presumably because their native state is perturbed. Reinsertion into a lipid bilayer can restore those activities (6). An early study utilized mixed lipid-detergent micelles to probe possible effects of lipids on receptor activity (5). However, there is no published information about effects on chemoreceptors of lipid composition in the natural environment of a membrane bilayer. We used Nanodiscs, small (~10-nm) plugs of lipid bilayer rendered water-soluble by an annulus of "membrane scaffold protein" (MSP)[2] (see Fig. 1*B*) to vary the composition of the bilayer into which the aspartate chemoreceptor Tar from *E. coli* was inserted and thereby investigate influences of membrane lipid environment on a chemoreceptor. Nanodiscs provided a convenient way to perform these studies because they can be made with many different lipids and lipid combinations (7). Furthermore, the relatively high ratio of protein to lipid in a Nanodisc creates a bilayer environment that mimics features of the natural, protein-dense cytoplasmic membrane (8–10).

Like all chemoreceptors for which the relevant information is available, the fundamental structural unit of *E. coli* Tar is a homodimer (2). This dimer is an extended helical structure (see Fig. 1*B*). On the periplasmic side of the cytoplasmic membrane, two antiparallel four-helix bundles interact to create ligand binding sites at their interface (11). For Tar and related chemoreceptors, two helices from each bundle extend through the membrane as a loose four-helix bundle (3, 4, 12). One helix from each subunit connects to a signal conversion segment, a HAMP domain that itself is a parallel four-helix bundle. Two HAMP domain helices extend to form two of the four helices of an extended four-helix coiled coil, which at its membrane-distal hairpin tip interacts with the signaling histidine kinase CheA and the coupling protein CheW to form signaling complexes

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[1] To whom correspondence should be addressed: Dept. of Biochemistry, 117 Schweitzer Hall, University of Missouri, Columbia, MO 65211. Tel.: 573-882-4845; Fax: 573-882-5635; E-mail: hazelbauerg@missouri.edu.

[2] The abbreviations used are: MSP, membrane scaffold protein; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol; PC, phosphatidylcholine; Hex II, hexagonal, non-bilayer organization of membrane lipids.

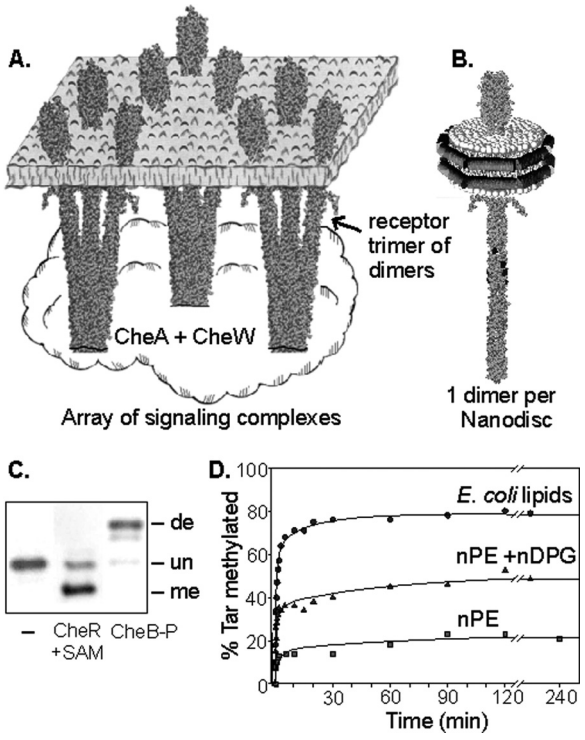

FIGURE 1. **Chemoreceptors and assessment of receptor adaptational modification.** *A*, graphic of a portion of an extended array of chemotaxis signaling complexes illustrating native membrane-embedded trimers of chemoreceptor dimers interacting with CheA and CheW (cloud-like region) at their distal signaling tips. *B*, graphic of a Nanodisc containing a single chemoreceptor homodimer. *C*, immunoblot of chemoreceptor Tar showing the relevant section of a SDS-polyacrylamide gel from which the extent of adaptational modification can be determined. From *left* to *right*, the gel lanes contained Tar that was untreated (−), treated with CheR plus the methyl donor *S*-adenosylmethionine (*CheR* + *SAM*), or treated with phosphorylated CheB (*CheB-P*). Labels on the *right* mark the positions of fully deamidated (*de*), unmodified (*un*), and fully methylated (*me*) Tar. *D*, representative time courses used to determine the plateau extents of Tar methylation for receptor inserted in Nanodiscs. In this case, Tar was inserted in membranes made with native (*E. coli* lipids), native phosphatidylethanolamine plus diphosphatidylglycerol (*nPE* + *nDPG*), or native phosphatidylethanolamine (*nPE*).

(see Fig. 1*A*). The bacterial chemosensory system adapts to persistent stimulation. The mechanism is covalent modification, methylation or demethylation for positive or negative stimulation, respectively, of four or five specific glutamyl residues on the surface of the extended cytoplasmic coiled coil (2). Methylation is catalyzed by methyltransferase CheR and demethylation by methylesterase CheB. CheB also catalyzes deamidation of specific chemoreceptor glutamines to create two of the methyl-accepting glutamyl residues in the mature protein (2).

When incorporated into Nanodiscs made with bulk phospholipid extracted from *E. coli* membranes, chemoreceptor Tar assumes a functionally native state that can exhibit the full range of receptor activities (6, 13–15). Thus bilayers contained in Nanodiscs are an appropriate environment in which to investigate effects of lipids on chemoreceptor structure. In the cell, chemoreceptors form trimers of dimers and extensive hexagonal arrays of signaling complexes (see Fig. 1*A*) (2, 16). To focus on effects of the lipid environment on the fundamental structural unit, the receptor dimer, and avoid possible complexities of dimer-dimer interactions, we studied dimers isolated from each other in individual Nanodiscs (see Fig. 1*B*). Such isolated dimers exhibit all chemoreceptor activities not dependent on trimer formation, including modification by the adaptational enzymes and transmembrane signaling (6, 13).

We assessed the extent to which each lipid environment supported functionally relevant Tar structure by assaying covalent modification by the enzymes of adaptational modification. Modification by these enzymes is a useful assay of receptor structure. Both enzymes are thought to clasp the four-helix, coiled-coil structure of the chemoreceptor cytoplasmic segment around a substantial fraction of its circumference, placing the active site near the side chain to be modified (17–19). Detergent-solubilized receptors are not modified by the enzymes (5, 6), presumably because perturbations of helical structure, packing, and/or dynamics disrupt productive clasping and thus catalysis. Incorporation of detergent-solubilized receptor into a Nanodisc-enclosed membrane formed from native *E. coli* lipids reverses this disruption, generating a functionally native chemoreceptor structure and thus almost complete modification by the adaptational enzymes (6, 13). The combination of Nanodisc technology and assays for functionally native states of receptors using adaptational modification allowed us to investigate influences of lipid bilayer environment on chemoreceptors.

EXPERIMENTAL PROCEDURES

Materials—Membrane scaffold protein MSP1D1(−) (20) and chemoreceptor Tar with a 6-histidine carboxyl-terminal extension (Tar-6H) plus a QEQE arrangement at the four methyl-accepting sites (21) were purified as described (22). A polar extract of total *E. coli* lipids, individual natural lipids purified from such an extract, and individual synthetic lipids were purchased in chloroform solutions from Avanti Polar Lipids (Alabaster, AL). Each synthetic lipid carried one species of fatty acid at the two fatty acid positions. These were as follows: phosphatidylethanolamine (PE) with exclusively saturated or unsaturated fatty acids had two 16:0 or 16:1 fatty acids, respectively; phosphatidylglycerol (PG) 16:0 or 18:1; and diphosphatidylglycerol (DPG) 14:0 or 18:1. The exclusively unsaturated fatty acids of monomethylated PE and of phosphatidylcholine (PC) were 18:1. Stock lipid solutions were prepared by taking the chloroform solution to dryness with a stream of nitrogen, addition to the dried lipid of a volume equivalent to the original chloroform solution of 50 mM Tris-HCl (pH 7.5) and cholate at 100–300 mM, agitation in a sonicating water bath until the mixture cleared (3–6 h), and storage at −80 °C under argon (22). Concentrations of lipids in these solutions were determined using a total phosphorous assay (23).

Nanodiscs—Nanodiscs were prepared essentially as described (22) using an MSP/lipid molar ratio of 1:60 and a Tar-6H/MSP ratio of 1:10. Receptor-containing Nanodiscs eluted from the nickel column were fractionated by size-exclusion chromatography on a TSK 5000 column to obtain preparations of almost exclusively one Tar dimer per disc (15) and stored at −80 °C. Nanodiscs made with MSP1D1(−) contain ~80 phospholipids per membrane leaflet (20). Insertion of one chemoreceptor dimer would be expected to reduce that number to ~68 lipids per leaflet. Nanodiscs containing the three major *E. coli* lipids were made by mixing purified or synthetic lipids in the proportions 71 mol % PE, 24 mol % PG, and 5 mol % DPG. These ratios

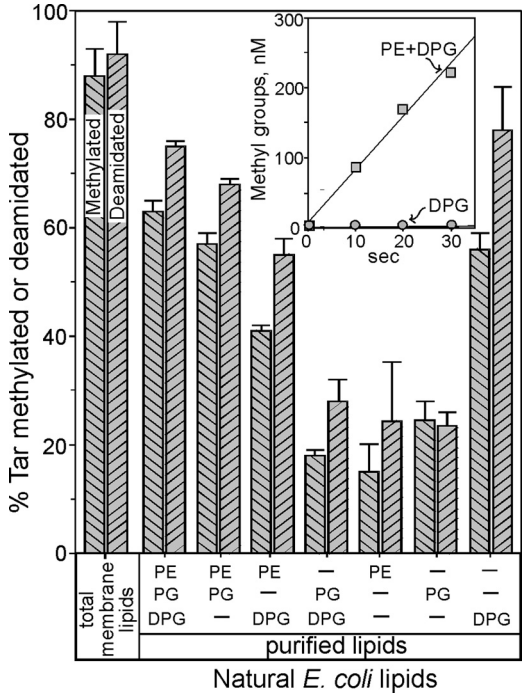

FIGURE 2. **Plateau extents of methylation and demethylation of Tar homodimers in Nanodiscs made with native *E. coli* phospholipids.** Bars show the plateau extent of methylation (*left-hand bar* of each pair, *stippling sloping down to the right*) and deamidation (*right-hand bar* of each pair, *stippling sloping down to the left*) in conditions designed to modify all receptors sufficiently native to be recognized by the respective enzymes (Fig. 1*D*). Values with *error bars* (standard deviations) are means of at least three independent experiments. The lipids from which the respective Nanodiscs were made are indicated along the abscissa: an unfractionated acidic extract of *E. coli* membrane (total membrane lipids) and combinations of one or more of the three major lipids of those membranes, PE, PG, and DPG, that had been purified from such extracts (purified lipids) and mixed at relative ratios representative of native *E. coli* membranes (see "Experimental Procedures"). The *inset* shows initial time courses of methylation for Tar inserted in Nanodiscs made with native phosphatidylethanolamine and diphosphatidylglycerol (*PE + DPG*) or native diphosphatidylglycerol (*DPG*).

corresponded essentially to the range of ratios determined for cytoplasmic membrane of *E. coli*: 70–75 mol % PE, 15–20 mol % PG, and 2–6 mol % DPG (24), and the relative proportions for the purchased polar extract of total *E. coli* lipids, 67:23:10. For mixtures of two lipids, we used ratios based on the relative proportions in the mixture of all three. For instance, we made mixtures of PG and DPG at a ratio of (24/29):(5/29) = 83:17.

Assaying Nanodisc-inserted Tar—Plateau extents of adaptational modification (see Fig. 1*D*) were produced by incubating 5 μM Nanodisc-inserted Tar for 2 h at room temperature with 5 μM CheR and 80 mM *S*-adenosylmethionine or with 5 μM CheB and 50 mM phosphoramidate (25, 26). Samples were analyzed by SDS-polyacrylamide gel electrophoresis, and gels were stained with Coomassie Brilliant Blue. Extents of methylation and deamidation were determined by densitometric quantification of the proportion of total Tar exhibiting electrophoretic mobility corresponding to mobility of the fully methylated or fully deamidated forms, respectively (see Fig. 1*C*). Initial rates of methylation were determined by incubating Nanodisc-inserted TarQEQE providing 2.5 μM available methyl-accepting sites with 0.125 μM CheR plus 50 μM *S*-adenosyl-L-[*methyl*-^{3}H]methionine and determining the extent of methylation at three times in the 40 s after the start of the reaction. We determined the extent of methylation using alkaline hydrolysis of excised bands of Tar from an SDS-polyacrylamide gel to yield radiolabeled methanol, vapor phase diffusion, and scintillation counting (13).

RESULTS

We incorporated chemoreceptor Tar into Nanodiscs made with different lipids. The receptor carried a six-histidine affinity tag at its carboxyl terminus and the native gene-encoded residues at its four sites of adaptational modification, *i.e.* two glutamates and two glutamines. The His_6 tag allowed separation of receptor-containing Nanodiscs from discs lacking Tar. Although the tag has subtle effects on the kinetics of modification, it has no detectable effects on the fundamental receptor activity of kinase activation or on the extent of receptor modification (21). Nanodiscs were prepared using an excess of scaffold protein relative to receptor to favor formation of discs containing a single chemoreceptor Tar dimer (6) and fractionated by size-exclusion chromatography to isolate Nanodisc-embedded receptors that were separated from aggregated or otherwise undesirable material and contained almost exclusively one Tar dimer per disc (14, 15). The four-helix bundle of the receptor transmembrane region would be expected to displace ~12 lipids from each membrane leaflet of a Nanodisc with a single inserted Tar dimer.

The state of isolated Tar dimers in different lipid environments was assessed by determining the proportion of Tar that had sufficiently native structure to be recognized and thus methylated by methyltransferase CheR as well as recognized and thus deamidated by methylesterase/deamidase CheB. Modifications were detected by altered migration of modified receptors in SDS-polyacrylamide gel electrophoresis (Fig. 1*C*) and assessed after a 2-h, room-temperature incubation with the respective enzyme at an equimolar concentration. These conditions generated plateau levels of modification (Fig. 1*D*) and thus identified the proportion of chemoreceptors that could attain the functionally relevant structural state. This parameter had a useful dynamic range because Tar removed from a lipid environment is essentially not modified (5, 6), and ~90% of receptors inserted in Nanodisc bilayers made from natural *E. coli* lipids are fully modified (Fig. 1*C*) (15). The ability to be modified has been used routinely to determine the proportion of accessible, functionally native receptors in isolated cytoplasmic membrane (27–29) or in Nanodisc preparations (6, 13, 15). However, plateau levels of receptor modification determined in conditions of extended incubation with equimolar enzyme would not necessarily be sensitive to relatively subtle structural perturbations. Thus for several lipid environments, we also determined initial rates of methylation in conditions of standard enzyme kinetics assays: low enzyme concentration relative to the substrate and time points in the first minute after initiation of the reaction (13, 30). For lipid environments in which plateau levels were low, determination of initial rates was not technically possible because of low absolute extents of methylation, but we considered a low plateau level of modification as sufficient for concluding that Tar was functionally non-native to the extent indicated in that environment. Nanodisc-embed-

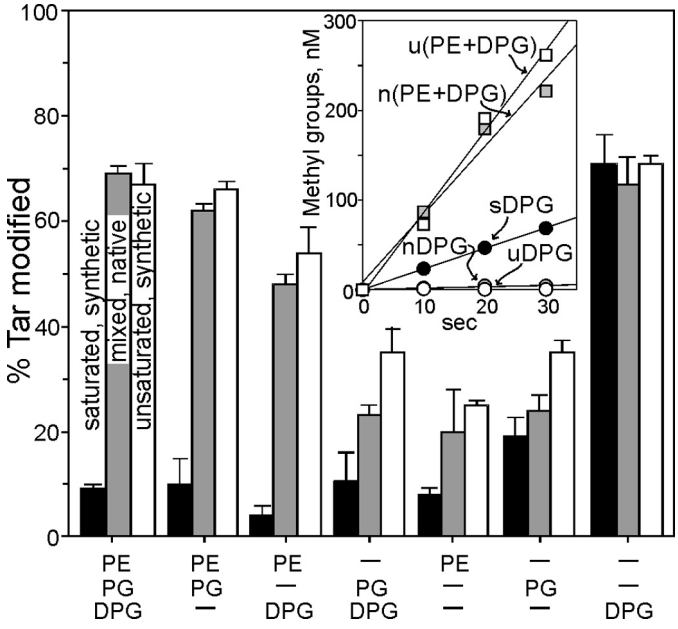

FIGURE 3. **Plateau extents of adaptational modification of Tar homodimers in Nanodiscs made with synthetic phospholipids.** Bars show the plateau extent of adaptational modification (the average of percent methylated and percent deamidated) for Tar inserted in lipid bilayers made with synthetic lipids carrying two saturated fatty acids (*left*, *black bar* of each triplet) or two monounsaturated fatty acids (*right*, *white bar* of each triplet) or purified from an extract of native *E. coli* membranes (*middle*, *gray bar* of each triplet; data from Fig. 2). Values with *error bars* (standard deviations) are means of at least three independent experiments. The lipids from which the respective Nanodiscs were made are indicated along the abscissa. The fatty acids carried by the synthetic lipids are listed under "Experimental Procedures." Combinations of lipids were prepared at their relative ratios in native *E. coli* membranes (see "Experimental Procedures"). The *inset* shows initial time courses of methylation for Tar inserted in Nanodiscs made with native phosphatidylethanolamine and diphosphatidylglycerol (*n(PE + DPG)*), native diphosphatidylglycerol (*nDPG*), synthetic phosphatidylethanolamine and diphosphatidylglycerol each carrying two monounsaturated fatty acids (*u(PE + DPG)*), or synthetic diphosphatidylglycerol carrying two saturated fatty acids (*sDPG*) or two monounsaturated fatty acids (*uDPG*).

ded Tar was considered in a functionally native state only if both plateau level of modification and initial rate of methylation were both a significant proportion of the values for Tar incorporated into Nanodiscs made with the natural lipid mixture extracted from native *E. coli* membranes.

Natural E. coli Lipids—Approximately 90% of Tar inserted into Nanodiscs made with a polar lipid extract of *E. coli* membranes was recognized and modified by CheR and by the active form of the deamidase, phospho-CheB (Fig. 2, *leftmost pair of bars*), confirming that insertion into a Nanodisc-enclosed lipid bilayer can provide a hydrophobic environment in which the receptor is in a functionally native state as defined by our assays (6). We made Tar-containing Nanodiscs using a mixture of the three major *E. coli* lipids, each of which had been purchased as an individual lipid purified from a polar extract and thus had the heterogeneity and distribution of fatty acids characteristic of natural *E. coli* membranes. The purified natural lipids were mixed in proportions representative of native membranes (24): 71 mol % PE, 24 mol % PG, and 5 mol % DPG. In these Nanodiscs, ~70% of inserted Tar was modified by the enzymes (Fig. 2, *second set of bars*). Thus bilayers reconstituted from purified natural lipids could generate a high proportion of functionally native receptor. This meant that we could use these purified lipids to vary lipid composition and thereby investigate the extent to which different bilayer features supported native receptor structure.

We prepared Tar-containing Nanodiscs with all possible combinations of one, two, or all three major lipids found in natural *E. coli* membranes: PE, PG, and DPG. For combinations of two lipids, the proportion of each was calculated using their respective relative abundance in the *E. coli* cytoplasmic membrane (see "Experimental Procedures"). Nanodiscs formed with the three natural lipids were the most effective in supporting functionally native receptors (Fig. 2). Elimination of the minority acidic lipid DPG hardly reduced the proportion of modified Tar; elimination of PG, the more prevalent of the two acidic lipids, modestly reduced the proportion; and elimination of the predominant, zwitterionic lipid PE substantially reduced the proportion modified. Tar in Nanodiscs containing PE or PG as the sole lipid exhibited low plateau levels of modification, indicating that little receptor was in a functionally native state. Receptor inserted into DPG-only membranes was also greatly perturbed, but in an unusual way. In assays of initial rate, performed in standard conditions of low enzyme concentration relative to substrate and sampling within the first minute, there was no detectable methylation of Tar in DPG-only bilayers. As an example, in Fig. 2, the *inset* contrasts the substantial initial rate of methylation for Tar inserted in a bilayer of PE + DPG to the undetectable rate for Tar inserted in a DPG-alone bilayer. We also observed comparably low initial rates of deamidation (data not shown). However, in conditions designed to identify all molecules in a receptor population that could possibly attain the enzyme-recognized structure, *i.e.* equimolar enzyme concentration and long incubation times, Tar in DPG-alone Nanodiscs became methylated or deamidated to an extent comparable with receptor in bilayers made from the mixture of all three *E. coli* lipids (Fig. 2, *far right pair of bars*). This unusual defective receptor state is considered further under the "Discussion."

Investigating Influences of Specific Lipid Features—The data in Fig. 2 demonstrated that Tar was sensitive to its lipid environment. However, many parameters contribute to that environment, and individual lipids can contribute in complex ways, in part because of variability and heterogeneity in fatty acid chains of natural lipids. To reduce this complexity, we utilized commercially available synthetic lipids in which both fatty acids were identical. This allowed us to compare lipid environments that differed by a more limited number of features. As illustrated in Fig. 2, all our experimental measurements of the extent of methylation and of deamidation for Tar in a specific lipid environment yielded sufficiently similar values for proportion of receptor in a functionally native state that the same conclusions about the relative effectiveness of that lipid composition would have been reached using either extent of methylation or extent of deamidation. Thus to simplify data presentation in Figs. 3, 4*A*, and 5*A*, we averaged methylation and deamidation plateaus to generate a value for "percentage of Tar modified" that is a representative parameter for the proportion of receptor in a functionally native state.

Saturated Versus Unsaturated Fatty Acids—We utilized synthetic PE, PG, and DPG, each carrying two identical saturated or two identical monounsaturated fatty acids, to prepare Tar-

containing Nanodiscs with the same headgroup combinations used in our study of natural lipids (see previous section). Because natural *E. coli* lipids contain approximately equal numbers of saturated and monounsaturated fatty acids (24), we could display data from Fig. 2 with data for synthetic lipids to compare effects of membranes with 0, ~50, or 100% unsaturated fatty acids (Fig. 3). Membranes made with lipids carrying 0 % unsaturated fatty acids resulted in a low proportion of functionally native Tar. For membranes made with two or three lipids, the presence of one unsaturated fatty acid chain per lipid substantially increased the proportion of modified receptor, and there were only modest, if any, additional increases if both fatty acids were unsaturated (Fig. 3). For membranes made exclusively with PE or PG, the presence of one or two unsaturated fatty acid chains resulted in small increases in the low proportion of functionally native Tar (Fig. 3). As in our characterization of effects of natural lipids, Tar in synthetic DPG-alone membranes was defective in assays for the initial rate of methylation but not the extent of modification. Initial rates were undetectably low when lipids carried one or two unsaturated fatty acids and substantially reduced with exclusively saturated fatty acids (Fig. 3, *inset*). In contrast, a high proportion of Tar inserted in DPG-alone Nanodiscs was modified in our assays of plateau levels of modification, independent of the proportion of unsaturated fatty acids. Given the unusual situation of Tar in DPG-only bilayers, the state of Tar in those membranes was not informative for identifying effects of fatty acid composition on chemoreceptors.

Phosphatidylethanolamine—We found that the presence of PE, the most prevalent lipid in *E. coli* membranes, was important for generating a substantial proportion of functionally native receptor structure. Fig. 4*A* displays relevant data from Figs. 2 and 3 to illustrate this effect. PE increased the proportion of functionally native structure whether the lipids carried the natural distribution of saturated and unsaturated fatty acids (*left-hand panel*) or exclusively unsaturated fatty acids (*right-hand panel*). The positive contribution of PE to generating native receptor structure was even more striking when assessed by the stringent assay of initial rate of methylation (Fig. 4*B*). In the standard conditions for that assay, Tar in membranes made from PG or from PG plus DPG exhibited no detectable initial rate of methylation, but Tar inserted into membranes that also included PE exhibited substantial rates.

Which feature or features of PE are important for supporting native chemoreceptor structure? PE has the propensity for forming non-bilayer Hex II structures instead of lamellar structures (31, 32), and its headgroup can form hydrogen bonds (33). At physiological pH, its zwitterionic headgroup has a net charge of ~0. To investigate the importance of Hex II formation, we prepared Tar Nanodiscs made with a mixture of the anionic lipid PG carrying two unsaturated fatty acid chains plus one of two different variants of PE that do not form Hex II but are still zwitterionic, neutral at physiological pH, and capable of forming hydrogen bonds. As assessed by the extent of modification or the initial rate of methylation, these variants, monomethylated PE carrying two unsaturated fatty acids or PE carrying two saturated fatty acids (32, 34), were at least as effective as natural PE in supporting functionally native Tar structure (Fig. 5*A*).

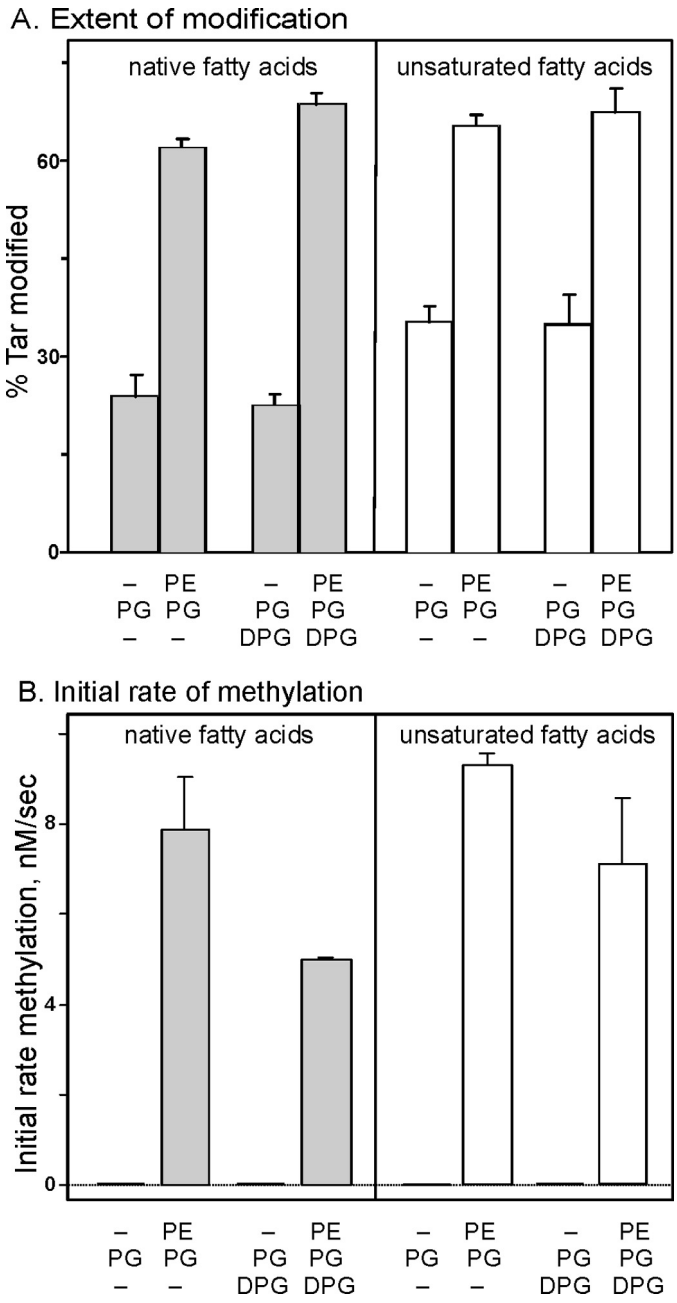

FIGURE 4. **Importance of phosphatidylethanolamine for functionally native receptor structure.** *A*, plateau extents of Tar modification in Nanodiscs lacking or containing PE. Data from Fig. 2 in the *left-hand panel* and Fig. 3 in the *right-hand panel* illustrate effects of the presence of PE on the proportion of Tar with the functionally native structure required for modification by the adaptation enzymes. *B*, initial rates of Tar methylation in Nanodiscs lacking or containing PE. In *A* and *B*, lipid compositions of the respective Nanodiscs are shown below each bar. Values with *error bars* (standard deviations) are means of at least three independent experiments.

Thus propensity to form the non-lamellar Hex II structure cannot be crucial for the positive effects of PE on the functional state of the receptor.

Next we investigated the importance of the hydrogen-bonding capacity of the amino group of PE. Trimethyl PE, *i.e.* PC, cannot make hydrogen bonds and does not have a propensity to form Hex II yet is zwitterionic and neutral at physiological pH. We made Tar Nanodiscs with synthetic DPG plus either PE or PC, using lipids that had a monounsaturated fatty acid at both

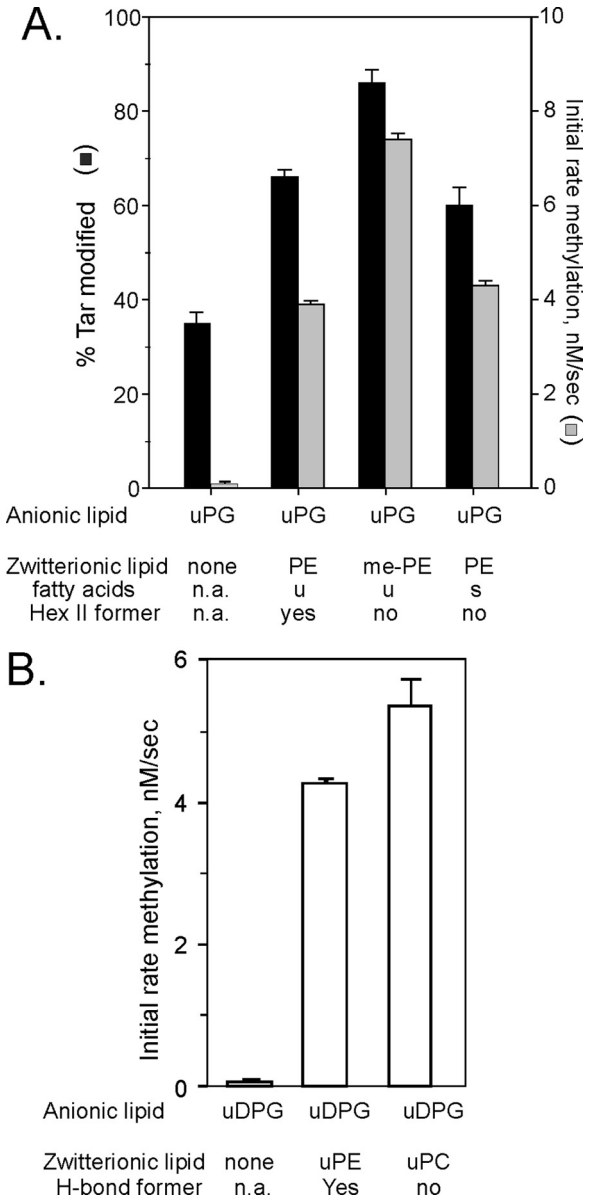

FIGURE 5. **Testing importance of specific properties of phosphatidylethanolamine for functionally native receptor structure.** *A*, propensity for Hex II, non-bilayer structures. Plateau extents of modification (*black bars*, *left-hand axis*) and initial rates of methylation (*gray bars*, *right-hand axis*) were determined for Tar in Nanodiscs made with the anionic synthetic lipid PG carrying two unsaturated fatty acids with no other lipid present (*uPG*) or with one of the following zwitterionic synthetic lipids: phosphatidylethanolamine carrying unsaturated fatty acids (*PE*) (strong propensity to form Hex II), monomethylated PE carrying unsaturated (*u*) fatty acids (*me-PE*) (little propensity to form Hex II), or PE carrying saturated (*s*) fatty acids (little propensity to form Hex II). The *bottom two lines* below the abscissa indicate the fatty acid composition and Hex II-forming ability of each PE form, respectively. *n.a.*, not applicable. *B*, formation of hydrogen bonds. Initial rates of methylation were determined for Tar in Nanodiscs made with only the anionic synthetic lipid DPG (*uDPG*) or with DPG plus a zwitterionic synthetic lipid: synthetic PE (*uPE*) (headgroup can form hydrogen bond) or synthetic trimethylated PE, *i.e.* phosphatidylcholine (*uPC*) (headgroup cannot form hydrogen bond). All lipids carried two unsaturated fatty acids. In *A* and *B*, values with *error bars* (standard deviations) are means of at least three independent experiments. The fatty acids of synthetic lipids are listed under "Experimental Procedures."

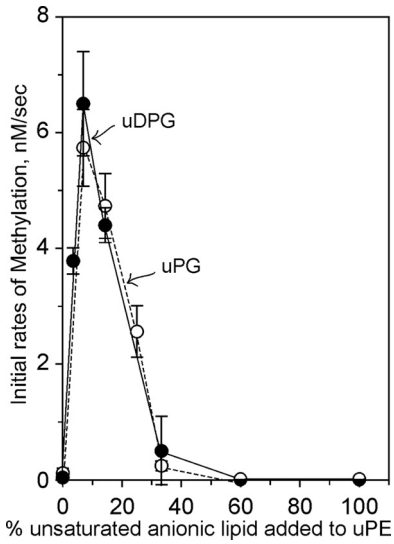

FIGURE 6. **Dependence of functionally native Tar on proportion of anionic lipid in Nanodisc membranes.** Initial rates of methylation were determined for Tar inserted into Nanodiscs made with the indicated mole percents of synthetic phosphatidylethanolamine (*uPE*) and phosphatidylglycerol (*uPG*) or diphosphatidylglycerol (*uDPG*), each carrying two unsaturated fatty acids. Values with *error bars* (standard deviations) are means of at least three independent experiments.

positions. As assayed by initial rates of methylation, PC was at least as good as PE in providing a membrane environment in which embedded Tar was effectively recognized and thus modified by the methyltransferase (Fig. 5*B*). This indicated that the hydrogen-bonding capacity of PE could not be the central contributor to generating functionally native receptor. By elimination, it appeared that the important feature is the zwitterionic, physiologically uncharged nature of PE.

Anionic Lipids—Although PE was important for native receptor structure, Tar inserted in PE-only membranes exhibited low extents of modification, no matter what the fatty acid composition (Figs. 2 and 3), as well as undetectable initial rates of methylation (see below). Generation of functionally native Tar required membranes containing both the most prevalent *E. coli* lipid, zwitterionic PE, and an anionic lipid: PG, DPG, or both (Figs. 2–4). We investigated the requirement for an anionic lipid by making Tar Nanodiscs with different proportions of PE *versus* an anionic lipid, PG or DPG, all lipids carrying two monounsaturated fatty acids, and assessing the functional state of the receptor using the initial rate of methylation (Fig. 6). Tar exhibited a measurable rate over a narrow range of anionic lipid content, implying that functionally native receptor structure was strongly influenced by the proportion of negative charge in its lipid environment.

DISCUSSION

This study provides the first information about the influence of the lipid composition of a membrane on the functional state of a chemoreceptor. It has long been known that detergent-solubilized chemoreceptors lacked most functional activities, presumably as the result of structural disruption (5, 6), and that reconstitution into a lipid membrane could restore function and thus a functionally native structure (6, 35). However, essentially nothing was known about the degree to which native structure was dependent on the nature of the lipid. We addressed this issue by reconstituting chemoreceptor Tar into membranes contained in Nanodiscs. In this initial study, we limited our investigation to the influence of the lipid environ-

ment on the fundamental chemoreceptor structural unit, the homodimer. We did so by preparing Nanodiscs containing only single Tar homodimers. These dimers were thus isolated from potential interactions with neighboring dimers in the same membrane. We found that the proportion of Tar in its functionally native state was strongly influenced by membrane lipid composition. Thus functionally native structure requires not only that the chemoreceptor transmembrane segment be surrounded by a hydrophobic lipid bilayer, but it also requires specific features of the lipids that create that environment. This dependence on particular lipid properties provides information important for designing and interpreting *in vitro* characterization of chemoreceptors and signaling complexes.

Our results indicate that chemoreceptor Tar is in large part tuned to its natural lipid environment, the *E. coli* cytoplasmic membrane. The highest proportion of functionally native receptor, as assessed by the extent and initial rate of adaptational modification, was in membranes formed from an acidic lipid extract of *E. coli* membranes (Fig. 2). This extract contained the three major lipids, PE, PG, and DPG, plus minor lipid constituents. A somewhat lower proportion of native Tar was found when receptor was inserted in membranes formed from the three major lipids that had been purified from the acidic extract and mixed in ratios reflecting the proportions found in native membranes (Fig. 2). The reduced efficiency implies that receptor structure is influenced, albeit modestly, by minor components of native membranes. The extent to which Tar is tuned to a native membrane environment was emphasized by effects of membranes made with fewer than all three of the major *E. coli* lipids. Nanodisc-enclosed bilayers made with one or two lipids were not as effective as all three, although eliminating DPG, present at only 5–10 mol % in native membranes, generated a very modest reduction in functional Tar (Fig. 2).

Effects of Fatty Acids—Natural lipids isolated from cell membranes carry a heterogeneous array of fatty acids. These fatty acids vary in chain length (primarily 16 and 18 carbons, with some two carbons shorter) and number of double bonds (zero, one, or two) with most lipids carrying one saturated and one monounsaturated fatty acid (24). Using chemically synthesized forms of the three major *E. coli* lipids, we found that the functionally native state of Tar was not dependent on this heterogeneity because synthetic lipids with two identical monounsaturated fatty acids were at least as effective at supporting native Tar structure as the natural lipids, no matter what the identity or combination of headgroups (Fig. 3). Use of synthetic lipids also provided information about the importance of unsaturated fatty acids for receptor structure. Independent of the polar headgroup, Tar embedded in membranes assembled from lipids with exclusively saturated fatty acids exhibited low levels of functionally native structure relative to membranes containing natural lipids, which carry ~50% unsaturated fatty acids (24), or synthetic lipids carrying 100% unsaturated fatty acids (Fig. 3). These observations could be taken to imply that lipid bilayer fluidity influences receptor structure. However, in Nanodiscs containing a receptor dimer, most lipid molecules are adjacent to, or one lipid away from, a protein surface. Thus interpreting results in terms of conventional notions of lipid fluidity may not be appropriate. In any case, at least one feature of exclusively unsaturated fatty acids in the lipid bilayer is unfavorable to generating functionally native receptor structure. It could be as simple as the inability to accommodate the uneven surface of the receptor by fatty acid side chains lacking the packing perturbations of a double bond.

Roles of Phosphatidylethanolamine and Ionic Lipids—Characterization of the extent of functionally native Tar in membranes containing different combinations of native lipids (Figs. 2 and 3) identified phosphatidylethanolamine, the predominant lipid in native *E. coli* membranes, as an important contributor to the generation of functionally native receptor structure (Fig. 4). Using synthetic lipids, some of which were not native to *E. coli*, we found that neither the properties of PE that favor non-bilayer Hex II structures nor its ability to hydrogen-bond were crucial for its ability to enhance the proportion of native Tar. By elimination, it appears that the zwitterionic, physiologically neutral nature of PE is the feature crucial for generating functionally native chemoreceptor.

We found that for lipids naturally found in *E. coli* membranes, functionally native Tar structure required both PE and an anionic lipid in its bilayer environment (Figs. 2 and 3), but too much negatively charged lipid was as deleterious as none at all (Fig. 6). Interestingly, a recent computational modeling study of a chemoreceptor inserted in membrane approximating the native one found evidence for clustering of anionic lipids near the receptor (36), an observation consistent with our finding that the presence of an anionic lipid is important for Tar. However, unlike that study, we did not observe any preference for DPG over PG. The requirement for some but not too much anionic lipid could reflect a need to modulate properties of PE such as its propensity for Hex II formation and/or for charge-charge interactions between positively charged Tar residues at the membrane interface and negatively charged bilayer lipids (37, 38). Whatever the need, it is optimally satisfied with only 7 mol % anionic lipid (Fig. 6). With ~68 lipids per Tar-containing disc (see "Experimental Procedures"), 7 mol % would correspond to ~5 anionic lipids per leaflet and ~10 per bilayer. The experimentally indistinguishable relationships between the mole percent of unsaturated PG or unsaturated DPG and generation of functionally native receptor, although DPG has two negative charges per molecule and PG has only one, implies that whatever the influential parameter, it is related to the number of anionic lipids per unit area, not the charge per unit area or a requirement for a specific anionic lipid.

The Unusual Case of Diphosphatidylglycerol—Tar inserted into DPG-only Nanodiscs exhibited apparently contradictory behavior in our two assays for functionally native receptor. Initial rates of methylation indicated that the receptor population was in large part nonfunctional, but plateau levels of modification implied that most of the population was functional. This apparent contradiction is likely to reflect the different features assessed by the two assays. Measurable initial rates require that a reasonable proportion of the receptor population is in a functional state during a brief period of exposure to a few enzymes relative to the receptor substrate. A substantial plateau extent of modification requires only that each modified receptor attain the functional state, even briefly, during the extended, 2-h incubation so that it can be recognized and modified by enzyme

present in as many copies as substrate. For instance, Tar in DPG-only bilayers could be in an equilibrium between a very probable abnormal state and an improbable but possible functionally normal state. In fact, results of mutational studies have led Parkinson and colleagues to suggest that chemoreceptors can be driven into an abnormal, nonfunctional state that is still in conformational equilibrium with functional states (39–41).[3]

Lipids and Chemoreceptors—In summary, our studies of effects of membrane lipids on chemoreceptor Tar indicate that the receptor is greatly influenced by the properties of those lipids and that in many ways the receptor is tuned to the features of its native lipid environment.

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REFERENCES

1. Zhulin, I. B. (2001) The superfamily of chemotaxis transducers: from physiology to genomics and back. *Adv. Microb. Physiol.* **45,** 157–198
2. Hazelbauer, G. L., Falke, J. J., and Parkinson, J. S. (2008) Bacterial chemoreceptors: high-performance signaling in networked arrays. *Trends Biochem. Sci.* **33,** 9–19
3. Barnakov, A., Altenbach, C., Barnakova, L., Hubbell, W. L., and Hazelbauer, G. L. (2002) Site-directed spin labeling of a bacterial chemoreceptor reveals a dynamic, loosely packed transmembrane domain. *Protein Sci.* **11,** 1472–1481
4. Peach, M. L., Hazelbauer, G. L., and Lybrand, T. P. (2002) Modeling the transmembrane domain of bacterial chemoreceptors. *Protein Sci.* **11,** 912–923
5. Bogonez, E., and Koshland, D. E., Jr. (1985) Solubilization of a vectorial transmembrane receptor in functional form: aspartate receptor of chemotaxis. *Proc. Natl. Acad. Sci. U.S.A.* **82,** 4891–4895
6. Boldog, T., Grimme, S., Li, M., Sligar, S. G., and Hazelbauer, G. L. (2006) Nanodiscs separate chemoreceptor oligomeric states and reveal their signaling properties. *Proc. Natl. Acad. Sci. U.S.A.* **103,** 11509–11514
7. Roos, C., Zocher, M., Müller, D., Münch, D., Schneider, T., Sahl, H.-G., Scholz, F., Wachtveitl, J., Ma, Y., Proverbio, D., Henrich, E., Dötsch, V., and Bernhard, F. (2012) Characterization of co-translationally formed nanodisc complexes with small multidrug transporters, proteorhodopsin and with the *E. coli* MraY translocase. *Biochim. Biophys. Acta* **1818,** 3098–3106
8. Shaw, A. W., McLean, M. A., and Sligar, S. G. (2004) Phospholipid phase transitions in homogeneous nanometer scale bilayer discs. *FEBS Lett.* **556,** 260–264
9. Denisov, I. G., McLean, M. A., Shaw, A. W., Grinkova, Y. V., and Sligar, S. G. (2005) Thermotropic phase transition in soluble nanoscale lipid bilayers. *J. Phys. Chem. B* **109,** 15580–15588
10. Kijac, A., Shih, A. Y., Nieuwkoop, A. J., Schulten, K., Sligar, S. G., and Rienstra, C. M. (2010) Lipid-protein correlations in nanoscale phospholipid bilayers determined by solid-state nuclear magnetic resonance. *Biochemistry* **49,** 9190–9198
11. Kim, K. K., Yokota, H., and Kim, S.-H. (1999) Four-helical-bundle structure of the cytoplasmic domain of a serine chemotaxis receptor. *Nature* **400,** 787–792
12. Boldog, T., and Hazelbauer, G. L. (2004) Accessibility of introduced cysteines in chemoreceptor transmembrane helices reveals boundaries interior to bracketing charged residues. *Protein Sci.* **13,** 1466–1475
13. Amin, D. N., and Hazelbauer, G. L. (2010) The chemoreceptor dimer is the unit of conformational coupling and transmembrane signaling. *J. Bacteriol.* **192,** 1193–1200
14. Li, M., and Hazelbauer, G. L. (2011) Core unit of chemotaxis signaling complexes. *Proc. Natl. Acad. Sci. U.S.A.* **108,** 9390–9395
15. Li, M., Khursigara, C. M., Subramaniam, S., and Hazelbauer, G. L. (2011) Chemotaxis kinase CheA is activated by three neighbouring chemoreceptor dimers as effectively as by receptor clusters. *Mol. Microbiol.* **79,** 677–685
16. Briegel, A., Li, X., Bilwes, A. M., Hughes, K. T., Jensen, G. J., and Crane, B. R. (2012) Bacterial chemoreceptor arrays are hexagonally packed trimers of receptor dimers networked by rings of kinase and coupling proteins. *Proc. Natl. Acad. Sci. U.S.A.* **109,** 3766–3771
17. Djordjevic, S., Goudreau, P. N., Xu, Q., Stock, A. M., and West, A. H. (1998) Structural basis for methylesterase CheB regulation by a phosphorylation-activated domain. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 1381–1386
18. Djordjevic, S., and Stock, A. M. (1997) Crystal structure of the chemotaxis receptor methyltransferase CheR suggests a conserved structural motif for binding *S*-adenosylmethionine. *Structure* **5,** 545–558
19. West, A. H., Martinez-Hackert, E., and Stock, A. M. (1995) Crystal structure of the catalytic domain of the chemotaxis receptor methylesterase, CheB. *J. Mol. Biol.* **250,** 276–290
20. Denisov, I. G., Grinkova, Y. V., Lazarides, A. A., and Sligar, S. G. (2004) Directed self-assembly of monodisperse phospholipid bilayer nanodiscs with controlled size. *J. Am. Chem. Soc.* **126,** 3477–3487
21. Lai, W.-C., and Hazelbauer, G. L. (2005) Carboxyl-terminal extensions beyond the conserved pentapeptide reduce rates of chemoreceptor adaptational modification. *J. Bacteriol.* **187,** 5115–5121
22. Boldog, T., Li, M., and Hazelbauer, G. L. (2007) Using Nanodiscs to create water-soluble transmembrane chemoreceptors inserted in lipid bilayers. *Methods Enzymol.* **423,** 317–335
23. Fiske, C. H., and Subbarow, Y. (1925) The colorimetric determination of phosphorus. *J. Biol. Chem.* **66,** 375–400
24. Morein, S., Andersson, A.-S., Rilfors, L., and Lindblom, G. (1996) Wild-type *Escherichia coli* cells regulate the membrane lipid composition in a window between gel and non-lamellar structures. *J. Biol. Chem.* **271,** 6801–6809
25. Barnakov, A. N., Barnakova, L. A., and Hazelbauer, G. L. (1998) Comparison *in vitro* of a high- and a low-abundance chemoreceptor of *Escherichia coli*: similar kinase activation but different methyl-accepting activities. *J. Bacteriol.* **180,** 6713–6718
26. Barnakov, A. N., Barnakova, L. A., and Hazelbauer, G. L. (1999) Efficient adaptational demethylation of chemoreceptors requires the same enzyme-docking site as efficient methylation. *Proc. Natl. Acad. Sci. U.S.A.* **96,** 10667–10672
27. Levit, M. N., and Stock, J. B. (2002) Receptor methylation controls the magnitude of stimulus-response coupling in bacterial chemotaxis. *J. Biol. Chem.* **277,** 36760–36765
28. Erbse, A. H., and Falke, J. J. (2009) The core signaling proteins of bacterial chemotaxis assemble to form an ultrastable complex. *Biochemistry* **48,** 6975–6987
29. Amin, D. N., and Hazelbauer, G. L. (2010) Chemoreceptors in signalling complexes: shifted conformation and asymmetric coupling. *Mol. Microbiol.* **78,** 1313–1323
30. Li, M., and Hazelbauer, G. L. (2005) Adaptational assistance in clusters of bacterial chemoreceptors. *Mol. Microbiol.* **56,** 1617–1626
31. van den Brink-van der Laan, E., Killian, J. A., and de Kruijff, B. (2004) Nonbilayer lipids affect peripheral and integral membrane proteins via changes in the lateral pressure profile. *Biochim. Biophys. Acta* **1666,** 275–288
32. Ellens, H., Bentz, J., and Szoka, F. C. (1986) Fusion of phosphatidylethanolamine-containing liposomes and mechanism of L_α-H_{II} phase transi-

[3] J. S. Parkinson, personal communication.

tion. *Biochemistry* **25,** 4141–4147
33. Murzyn, K., Róg, T., and Pasenkiewicz-Gierula, M. (2005) Phosphatidylethanolamine-phosphatidylglycerol bilayer as a model of the inner bacterial membrane. *Biophys. J.* **88,** 1091–1103
34. Mulukutla, S., and Shipley, G. G. (1984) Structure and thermotropic properties of phosphatidylethanolamine and its *N*-methyl derivatives. *Biochemistry* **23,** 2514–2519
35. Ninfa, E. G., Stock, A., Mowbray, S., and Stock, J. (1991) Reconstitution of the bacterial chemotaxis signal transduction system from purified components. *J. Biol. Chem.* **266,** 9764–9770
36. Hall, B. A., Armitage, J. P., and Sansom, M. S. (2012) Mechanism of bacterial signal transduction revealed by molecular dynamics of Tsr dimers and trimers of dimers in lipid vesicles. *PLoS Comput. Biol.* **8,** e1002685
37. Miller, A. S., and Falke, J. J. (2004) Side chains at the membrane-water interface modulate the signaling state of a transmembrane receptor. *Biochemistry* **43,** 1763–1770
38. Draheim, R. R., Bormans, A. F., Lai, R.-Z., and Manson, M. D. (2006) Tuning a bacterial chemoreceptor with protein-membrane interactions. *Biochemistry* **45,** 14655–14664
39. Parkinson, J. S. (2010) Signaling mechanisms of HAMP domains in chemoreceptors and sensor kinases. *Annu. Rev. Microbiol.* **64,** 101–122
40. Zhou, Q., Ames, P., and Parkinson, J. S. (2011) Biphasic control logic of HAMP domain signalling in the *Escherichia coli* serine chemoreceptor. *Mol. Microbiol.* **80,** 596–611
41. Kitanovic, S., Ames, P., and Parkinson, J. S. (2011) Mutational analysis of the control cable that mediates transmembrane signaling in the *Escherichia coli* serine chemoreceptor. *J. Bacteriol.* **193,** 5062–5072
42. Bogdanov, M., Heacock, P., Guan, Z., and Dowhan, W. (2010) Plasticity of lipid-protein interactions in the function and topogenesis of the membrane protein lactose permease from *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **107,** 15057–15062